 doi:10.20944/preprints201812.0362.v1

# An update on plant-derived compounds as potential inhibitors of the bacterial efflux pumps: with reference to *Staphylococcus aureus* and *Escherichia coli*

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**Short title:** Essential oils as a potential inhibitor of bacterial efflux pumps.

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 doi:10.20944/preprints201812.0362.v1

**Abstract**

Bacterial antibiotic resistance has become a major global health concern. One of the main reasons for the development of multi-drug resistance properties in bacteria is due to the bacterial efflux pump systems. They are important transport proteins, mainly involved in the removal of toxic substrates like antibiotics from inner cell environment. These pumps are responsible for the intrinsic ability of bacteria to get resistant to the antibiotic. Various types of efflux pumps are present in the Gram-positive and Gram-negative bacteria. Plant-derived products like Capsaicin, Olympicin A, and Indirubicin were found to be inhibitors of an efflux pump in *Staphylococcus aureus* similarly Ursolic acid derivatives; Daidzein and Lanatoside C were plant-derived inhibitors of an efflux pump in *Escherichia coli*. In this review detail information have been provided about efflux pump inhibitors that have been found to be effective in the Gram-positive bacteria and Gram-negative bacteria. The aim of this review is to focus on the role of plant-derived compounds as effective efflux pumps inhibitors with reference to mainly *Staphylococcus aureus* and *Escherichia coli*.

**Keywords:** Antibiotic resistance, efflux pump inhibitors, *Escherichia coli*, efflux pumps, multidrug resistance, *Staphylococcus aureus*

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## 1. **Introduction**

Bacterial infection has been considered to have a significant contribution towards the gradually increasing load of global infectious diseases and hence creating a havoc in human welfare and world economy [1]. Infections caused by deadly microbial agents have raised an alarm with increasing rates of morbidity and mortality throughout the globe. An estimated percentage of 50-75, hospital deaths have been reported to be caused by infectious diseases throughout the world [2, 3] and the number is still increasing. The prime reason behind this increasing rate of mortality is resistance developed by different pathogenic bacteria against the existing antibiotic drugs [4]. At present, antibacterial resistance has received utmost attention as a major concern of global public health by threatening the efficacy of existing antibacterial therapy and challenging the research for developing novel antibacterial [5]. Multidrug resistance (MDR) in different bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, etc. is developed by the mechanism of action of efflux pumps possessed by the MDR strains. Efflux pumps are a class of membrane-integrated proteins which play a crucial role in removal of toxic agents such as biocides, antibiotics, and toxic metals from bacterial cells to outer environment [6] and are classified into five different families (Table 1) such as Major facilitator super (MFS) family, Multidrug and toxic efflux (MATE) family, Resistance nodulation division (RND) family, Small multidrug resistance (SMR) family and ATP binding cassette (ABC) family. The phenomenon of multidrug resistance was first reported in *Enterobacteria* in the late 1950s [7]. A bacterium develops resistance towards antibiotics by different mechanisms like (i) modification or mutation in the antimicrobial target site for which the drug cannot recognize the respective site [8], (ii) producing drug-inactivating enzymes, which neutralize the effect of antibiotics [8], (iii) modification of cell wall protein which resists entry of drug (iv) activation of efflux pumps in bacterial cells [8]. *Staphylococcus aureus* is known for its mild to severe life-threatening infections [9, 10] and it exhibits a notable diversity of resistance towards various antimicrobial agents besides its virulence [11]. Tetracyclin pump is the first discovered efflux pump in 1980, found in *Escherichia coli* [12]. As of now, the presence of 15 different efflux pumps in *Staphylococcus aureus* have been recognized which are encoded either by the main chromosome or plasmid [6], whereas 29 efflux pumps have been identified in *Escherichia coli* [13]. These proteins are present both in antibiotic-resistant and antibiotic susceptible bacteria [14].

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The rapidly emerging trait of multidrug resistance in human pathogenic microorganisms against existing first line of drugs has raised a concern for the development of new antimicrobials from alternative sources including plants [3, 15]. Various plants have been recognized as medicinally important since mankind emerged and practiced because they harbor naturally active phytochemicals exhibiting significant antimicrobial activity with less toxicity. Numbers of phytochemicals like Porphyrin pheophorbide, silybin, methoxylated flavones, and isoflavone have been identified and listed for their effective synergistic activity against NorA efflux pump of *Staphylococcus aureus* strains [16-18]. These compounds are efficient efflux pump inhibitors of Gram-positive bacteria particularly *Staphylococcus aureus* but fail against Gram-negative bacterial strains such as *P. aeruginosa, E. coli*, and *Acinetobacter* as they contain thick, lipophilic outer membrane [18, 19]. Thus, primarily the bacterial cell membranes can be targeted for development of new antimicrobial agents [3]. Plant-derived secondary metabolites containing a steroid or triterpenoid aglycon attached to one or more sugar chains have been found to demonstrate cell membrane permeabilizing properties [3, 20].

During the development of an effective and efficient drug molecule, the most important aspect to focus on is an accumulation of sufficient knowledge on detailed structures of the substrate molecules and their functional behavior. This review emphasizes on different structural attributes of efflux pumps and their mode of action on different substrates extrusion. Till date, different plant-derived compounds have been reported to found effectively inhibit the function of bacterial efflux pumps. In this review, a detailed update has been presented about identified phytochemicals and their source along with nature of the action on different efflux pumps.

## 2. Bacterial efflux pumps

To avoid intracellular accumulation of toxic compounds, bacteria evolved an energy-dependent mechanism to expel such molecules from the cell via Efflux pumps and this process does not involve any alteration or degradation of the drugs [7]. Efflux pumps are found in almost all bacterial species belonging to Gram-positive and Gram-negative classes as well as in eukaryotic organisms [21] and the genes encoding this group of proteins can be located on plasmids or the chromosomes [14, 22, 23]. But the composition of efflux pump is different in both Gram-positive and in Gram-negative bacteria. In Gram-positive bacteria efflux pump are build up generally by a single polypeptide located in the cytoplasmic membrane [7] whereas in Gram-

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negative bacteria, efflux pumps are structured with three elements namely (i) inner membrane pump protein containing 12 transmembrane regions, (ii) two large periplasmic loops called membrane fusion proteins and (iii) an outer membrane protein which forms channel tunnel [7]. These pumps may be specific for each substrate or may transport a range of structurally disparate compounds including multiple classes of antibiotics. Such pumps are associated with Multi-Drug Resistance (MDR). Overexpression of efflux pump is responsible for the development of MDR in the bacterial cell [24]. Another mechanism of multidrug resistance development is amino acid substitution in efflux protein which enhances the export process [25].

Efflux pump also plays an important role in the physiological and metabolic activity of bacteria such as excretion of toxic metabolites, stress adaptation, development, survival, pathogenesis, and virulence in bacteria [5, 23, 26]. Expression of the Efflux systems is controlled by different local and global regulatory pathways [7].

Efflux pumps are incorporated in the class of active transporters as they move substrate against their electrochemical gradient and require input of energy. Mainly these active transporters are divided into two classes; (a) Primary active transporter- They obtain their required energy when there is a change in the chemical state of one of the reactants. ABC-transporters belong to this class as they utilize energy from the ATP hydrolysis and (b) Secondary active transporter- Except ABC transporters all transporter family belongs to the secondary active transporter, who transport molecules through secondary active transport mechanism. The secondary active transport mechanism is also known as coupled transport or co-transport in which energy is provided by electrochemical potential difference, created by the pumping of ions in or out of the cell. In this case, pumps act as symports or antiports, coupling the drug efflux to the downhill transport of an ion along a concentration gradient [27]. Symports- Is an integral membrane protein that is involved in the transport of many different types of molecules across the cell membrane but antiport simultaneously transports two different molecules across the membrane in opposite directions. All these transporter families utilize proton motive force as energy source except ABC transporter family, which utilizes adenosine triphosphate (ATP) hydrolysis for their export of substrates [28].

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## 2.1 Efflux pump in *Staphylococcus aureus*

The fight against infectious and deadly microorganisms using the first line of available defense systems primarily use of antibiotic drugs has been depressingly affected by the emergence of multidrug-resistant bacteria (MDR). As previously mentioned, *Staphylococcus aureus*, Gram-positive cocci, positions among the major bacterial pathogens responsible for mild to severe life-threatening infections [9, 10]. Mainly, Methicillin-resistant *Staphylococcus aureus* (MRSA), resistant to all beta-lactam antibiotics, is the major worry which has been reported for major outbreaks in nosocomial environments and at an increasing rate are now being isolated from the community where they may cause severe and deadly infections [10, 29]. Four distinct families of Efflux pumps belong to Gram-positive bacteria namely major facilitator superfamily (MFS), small multi-drug resistance (SMR), multi-drug and toxic compound extrusion (MATE), and ATP- binding cassette (ABC) [30] and the most significant and characteristic transporters of Gram-negative bacteria belong to the resistance-nodulation-cell division (RND) superfamily [30]. A large number of transporter proteins found in Gram-positive bacteria belong to Major facilitator superfamily (MFS) which composed of approximately 400 amino acids organized in 12 or 14 transmembrane helices with a large cytoplasmic loop among six and seven helices [30]. SMR transporter family proteins consist of about 110 amino acid residues with four transmembrane segments [30]. Proteins belonging to ABC transporter family contains four domains: two nucleotide binding domain (NBD) and two transmembrane domain (TMD) [30, 31]. The TMDs contain six transmembrane α-helices and form homo- or heterodimers. The NBDs bind to ATP molecule on the cytoplasmic end and interact with transmembrane domains [30, 32]. Multi-drug and toxic compound extrusion (MATE) efflux proteins consist of 400–700 amino acids that form 12 trans-membrane helices. All MATE family proteins exhibit nearly 40% identity of their amino acid sequence. All genes which encode MATE proteins are derived from the identical gene which was subsequently duplicated. An example of multi-drug and toxic compound extrusion (MATE) efflux pump in Gram-positive bacteria is MepA which is found in *Staphylococcus aureus* [33]. Many studies have revealed that increased resistance to antibiotics, various dyes and biocides has been mainly connected to NorA and NorB Efflux pumps [34-40]. Over the last decade, all research efforts are focused on the efflux pump NorA (MFS family) in *Staphylococcus aureus* [34]. However, the antimicrobial resistance associated with these two pumps is poorly characterized in *Staphylococcus aureus* [34, 35]. The chemical structures of

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NorA efflux pump inhibitors have been identified. It belongs to those families of compounds which hold conjugated double bonds. For example, chalcones, piperine like compound, citral amide derivatives or N-cinnamoylphenalkylamides. Indole, dihydronaphthyl-, 2-chloro-5-bromo-phenyl- or piperidine moieties appear to be beneficial for the efflux pump inhibitory properties [34].

Different Efflux pumps reported to be present in *Staphylococcus aureus* have been listed in Table 2.

**2.1.1 QacA and QacB**

QacA and QacB are members of Major Facilitator Superfamily **(**MFS). QacA Efflux pump was first identified on plasmid pSK1 in *Staphylococcus aureus* in the early 1980s [10, 41]. It is composed of 514 amino acids, presenting 14 transmembrane segments [42]. On the other hand, *QacB* efflux was first detected in the early 1950s on DNA plasmids and particularly on plasmid pSK23 [43]. Both QacA and QacB transporters rely on proton motive force via an antiport $H^+$: drug mechanism for drug extrusion from the bacterial cell [41, 44, 45]. The gene expression of both *QacA* and *QacB* is regulated by QacR [46-50]. Both QacA and QacB genes are closely related and differ from each other only by seven nucleotides. QacB confer high resistance to monovalent dyes [43, 45, 47] whereas QacA mediates resistance to both mono- and divalent cations which include dyes such as acriflavine, ethidium bromide, quaternary ammonium compounds (QACs), divalent cations such as propamidine isethionate, biguanidines and diamidinodiphenylamine dihydrochloride, etc. [45-47].

**2.1.2 LmrS**

LmrS (Lincomycin resistance protein of *S. aureus*) is one proton-coupled multidrug antiporter efflux pump containing 480 amino acids with 14 transmembrane domains and belongs to Major Facilitator Superfamily (MFS). It shares 39% identity with LmrB of *B. subtilis*, 25% identity with FarB protein *N. gonorrhoeae* and EmrB of *E. coli* [51]. This efflux pump is able to extrude lincomycin, kanamycin, linezolid, tetraphenylphosphonium chloride, sodium dodecyl sulfate, trimethoprim, and chloramphenicol, etc [51, 52].

**2.1.3 MdeA**

The MdeA (Multidrug Efflux Pump) transporter protein is composed of 479 amino acids [52, 53] with 14 predicted transmembrane segments. It is a member of Major Facilitator Superfamily (MFS) bearing molecular weight of 52 kDa and encoded by a chromosomal gene mdeA [53].

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This transporter protein utilizes proton motive force as an energy source to transport its substrates [10]. It shares 23% identity with QacA Efflux pump from *S. aureus*, 24% with an EmrB pump from *E. coli* and 37% similarity with Efflux pump LmrB from *B. subtilis* [53, 54]. MdeA exhibit resistance on *Staphylococcus aureus* to a range of quaternary ammonium compounds (benzalkonium chloride, dequalinium), dyes (Ethidium Bromide) and antibiotics (virginiamycin, novobiocin, mupirocin and fusidic acid) but not to fluoroquinolones, norfloxacin and ciprofloxacin [53, 55].

**2.1.4 QacG, QacH and QacJ**

QacG, QacH, and QacJ efflux pumps are encoded by plasmid genes and belong to Small Multidrug Resistance (SMR) family. The primary amino acid sequences of QacJ, QacG, and QacH are similar to each other [52, 56]. These three Efflux pumps are known to confer resistance to antiseptics and disinfectants in *S. aureus* [10]. The gene encoding for QacG pump resides on a 2.3 kb plasmid pST94 [10, 52]. QacG contains 107 amino acids and four transmembrane segments, which shares 69.2% identity with Smr Efflux pump belonging to SMR family of transporters [57]. QacH transporter protein with 107 amino acid residues and four transmembrane segments was first identified in 1998 by Heir and colleagues on a 2.4 kb plasmid (p2H6) and its gene shares 76% and 70% nucleotide similarity to the SMR and QacG genes, respectively [58]. QacH and QacJ transporter proteins also belong to SMR family. QacJ gene was identified on a 2.65 kb plasmid (pNVH01) [56] which shares 72.5%, 82.6% and 73.4% identity with the efflux pumps Smr, QacG, and QacH, respectively [10]. The protein composed of 107 amino acids and four transmembrane segments. All the three transporter proteins have been identified to confer similar levels of resistance to benzalkonium chloride, ethidium bromide and cetyltrimethylammonium bromide [10, 56].

**2.1.5 NorA**

NorA is one of the most studied efflux systems in *S. aureus*. The pump with molecular weight 42.3 kDa comprises of 388 amino acids, possesses 12 transmembrane segments and belongs to Major Facilitator Superfamily [10]. NorA gene was first identified in fluoroquinolone-resistant isolate *S. aureus* TK2566 collected in 1986 at a Japanese hospital [59]. Displaying some genetic diversity, the three norA alleles described till date possess up to 10% difference in their nucleotide sequences [60-62]. It shares 44% and 24% identity with Bmr multidrug efflux pump from *B. subtilis* and the tetracycline efflux pump Tet(A) from *E. coli*, respectively [63, 64].

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NorA exhibits extrusion of substrates via an $H^+$: drug antiport mechanism using proton motive force to energize the transport machinery [10]. NorG acts as an activator for NorA gene expression [65], whereas MgrA has been reported to negatively regulate expression of NorA gene [66]. NorA is responsible for a resistance for hydrophilic fluoroquinolones (norfloxacin, ciprofloxacin), monocationic compound (Ethidium bromide, Tetraphenylphosphonium bromide) and antiseptics (benzalkonium chloride, acriflavine, cetrimide) [67] but not resistant for lipophilic fluoroquinolones (sparfloxacin, moxifloxacin) [63]. NorA homologous pumps can be considered to be related with Efflux pumps of some other bacterial species such as EmeA (Enterococcal multidrug resistance efflux) pump from *E. faecalis* [68] and PmrA (Pneumococcal multidrug resistance protein) pump of *Streptococcus pneumoniae* [69]. NorA has been found to be responsible for the occurrence of MDR- type resistance due to overexpression of the Efflux pump.

**2.1.6 NorB, NorC and NorD**

NorB, NorC and NorD efflux pumps belonging to the Major Facilitator Superfamily (MFS). NorB is a chromosomally encoded efflux pump which contains 463 residues of an amino acid with 12 transmembrane segments [10]. The *norB* gene is 1392 bp long and encodes with a 49 kDa protein [52]. It confers resistance to some of the NorA substrates, such as biocides (tetraphenylphosphonium and cetrimide), hydrophilic fluoroquinolones (norfloxacin and ciprofloxacin), and dye (ethidium bromide), as well as to non-NorA substrates, such as the hydrophobic fluoroquinolones (moxifloxacin, sparfloxacin), and tetracycline [37]. NorB has been identified to possess a putative role in Staphylococcal pathogenesis [40]. The pump shares 41%, 30% and 39% structural similarity to Blt Efflux pump from *B. subtilis*, NorA pump from *S. aureus* and QacA from *S. aureus*, respectively [10].

NorC efflux pump consisting of 462 amino acid and 14 transmembrane segments [70] shares 61% identity with NorB [52]. NorC confers resistance to quinolones such as ciprofloxacin, norfloxacin, sparfloxacin, moxifloxacin, garenoxacin, and dye rhodamine [10, 70, 71].

NorD is a more recently studied chromosomally encoded Efflux pump belongs to the Major Facilitator Superfamily (MFS) and composed of 12 membrane domains [52].

**2.1.7 TetA(K) and Tet38**

Tet(K) or TetA(K) and Tet38 are plasmids encoded Tetracycline Efflux pumps which belong to the Major Facilitator Superfamily (MFS). TetA(K) antiporter protein, bearing molecular weight

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7 kDa, consists of 459 amino acids residues [52, 72] and 14 transmembrane segments. TetA(K) confers a high level of resistance to tetracycline, oxytetracycline, and chlortetracycline, however, less resistance is observed for antibiotic drugs like minocycline, 6-demethyl-6-deoxytetracycline and doxycycline [52]. Tet38, encoded by *tet38* gene of 1353 bp, is a 19 kDa protein containing 450 amino acid residues with 14 transmembrane domains. *tet38* gene is negatively regulated by MgrA [37], but NorG does not possess any role of binding the control elements for the gene [10]. *tet38* gene expression leads to 32 –fold increase in the resistance to tetracycline but not for other drugs like minocycline. It shares 46% similarity with TetA(K) from *S. aureus* and 45% identity with TetA(L) from *B. subtilis* [34, 37].

**2.1.8 SdrM, Mef(A) and SepA**

Both SdrM and Mef (A) efflux pumps belong to Major Facilitator Superfamily (MFS) whereas SepA is a member of Small Multidrug Resistance (SMR) family. SdrM is an energy-dependent multidrug efflux pump, encoded by the *sdrM* gene, possess 14 transmembrane segments [52]. It shares 23% and 21% of NorB and QacA Efflux pumps from *S. aureus*, respectively [10]. This kind of pump provides a low level of resistance to antimicrobial agents like acriflavine, ethidium bromide, fluoroquinolone, and norfloxacin [10, 52]. MgrA regulates the expression of the chromosomal gene *sdrM* [52].

Mef(A) is a chromosomally encoded multidrug efflux pump and was first identified in *Streptococcus pyogenes* in 1996 [73]. These transporters play role in the active extrusion of macrolides but cannot efflux out lincosamides and streptogramins [52, 74].

SepA protein is also a chromosomally encoded efflux protein consisting of 157 amino acids and four putative transmembrane segments [10]. It exhibits low-level resistance towards antiseptic compounds, namely chlorhexidine gluconate , benzalkonium chloride, and the dye acriflavine [10, 75].

**2.1.9 MepA**

MepA (Multidrug Export Protein) is the first multidrug transporter identified in *S. aureus* norA disrupted mutants [76] that belongs to MATE family [10]. It comprises of 451 amino acid residues, possesses 12 transmembrane segments and exhibits 26% and 21% similarity to the MATE transporters CdeA from Clostridium difficile and NorM from *Vibrio parahaemolyticus*, respectively. The pump is identified to provide low resistance to quaternary ammonium compounds (benzalkonium chloride, cetrimide, dequalinium, tetraphenylphosphonium,

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pentamidine) and dye (ethidium bromide), antibiotic (tigecycline) [10]. The fluoroquinolones norfloxacin and ciprofloxacin were reported to be weak substrates of MepA [10, 76, 77].

## 2.2 Efflux pump in *Escherichia coli*

*Escherichia coli* are recognized as the most commonly studied prokaryotic microorganism and belong to the family *Enterobacteriaceae*. It is a Gram-negative, facultatively anaerobic, rod-shaped bacterium. It lives in the human gut as a commensal microorganism but is also found to be involved in pathogenesis causing severe infections such as urinary tract infections (UTIs), sepsis, etc [78]. *E. coli* is categorized into six different pathotypes: enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *Escherichia coli* (EHEC), enteroinvasive *Escherichia coli* (EIEC), enteroaggregative *Escherichia coli* (EAEC) and the diffusely adherent *Escherichia coli* (DAEC) [52]. In *E. coli*, 1 MATE, 1 ABC, 1 SMR, 6 RND, and 7 MFS transporters have been reported to perform extrusion activity of antibiotics and conferring resistance to various drugs upon overexpression from plasmid-cloned genes. Drug resistance is mediated by several mechanisms in *Escherichia coli*. But one of the chief mechanisms for the development of resistance in these bacteria is the overexpression of AcrAB efflux pump [79]. Efflux pump AcrAB-TolC serves as an important antibiotic resistance and plays a major role in the multi-drug resistance phenotype of *Escherichia coli* clinical isolates [23, 80, 81]. AcrAB-TolC, AcrEF-TolC, and EmrE from *Escherichia coli* are positioned among the best-studied biocide extruding systems [82-84]. A list of Efflux pumps present in *E. coli* has been documented in **Table 2**.

### 2.2.1 AcrAB-TolC

AcrAB Efflux pump belongs to RND (Resistance Nodulation Division) family and specific to only Gram-negative bacteria [85]. It is composed of 1049 amino acid residues [86]. In *E. coli*, AcrAB-TolC pump provides resistance to bile salts [79]. Studies have also revealed that AcrAB-TolC efflux pump confers resistance against fluoroquinolone and other antimicrobials [87]. AcrAB pump, cooperating with TolC, causes extrusion of some more substrates such as dyes, detergents, tetracyclins, chloramphenicol, β-lactams, macrolides, and organic solvents [88, 89]. A periplasmic protein AcrA links an outer membrane channel TolC and secondary active transporter AcrB of inner membrane in AcrAB-TolC efflux pump [88]. Expression of the pump

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is negatively regulated by AcrR [81, 84] and positively regulated by three XylS/AraC family regulators [84, 90], MarA, SoxS, and Rob [84].

**2.2.2 AcrAD-TolC**

AcrAD is another RND- type Efflux pump, encoded by the *acrD* gene, found in *E. coli* which play a similar role to AcrB and responsible for extrusion of antiseptics such as Acriflavine [91]. It consists of 1,037 amino acids and shares 66.1% similarity with AcrB [91]. AcrAD-TolC mainly causes expulsion of aminoglycosides [91, 92], β-lactams [91, 93]and weak specificity for SDS, cholic acid, deoxycholate, novobiocin [91, 94]

**2.2.3 AcrEF-TolC**

AcrEF is an Acriflavine efflux pump and belongs to RND family [91]. The protein is encoded in an operon which contains RND *acrF* and MFP *acrE* gene and is regulated by *sdiA* [91, 95]. In this pump, the subunit AcrE contains 385 amino acids and shares 69.3% homology with AcrA whereas AcrF has 1,034 amino acids and shares 77.6% identity with AcrB [91].

**2.2.4 MdtABC-TolC**

MdtABC-TolC (Multidrug Transport) is encoded in a single operon and the expression is regulated by BaeSR [91, 96]. The *mdtABC* genes encode an RND system which contains RND pump genes, *mdtB* and *mdtC* and an adaptor gene *mdtA* [97]. According to reports, in *E. coli*, these transporters play a major role in export of Enterobactin [98]. MdtABC-TolC causes extrusion of several other substrates such as novobiocin [91], quinolones, fosfomycin, detergents [91, 94], zinc [99], myricetin [100] and bile salts [96].

**2.2.5 MdtEF-TolC**

MdtEF-TolC is another important multidrug transport efflux system which exhibits resistance against nafcillin, cloxacillin, oxacillin, erythromycin, rhodamine 6G, and SDS under the regulation of GadX [91, 101]. The genes for the Efflux system are located in and operon *gadX* [101], *gadY* [102] and *gadE* [103]. Studies have revealed that MdtEF is up-regulated under anaerobic growth condition of *E. coli* [104]. The proteins MdtE and MdtF share 55% and 71% similarity with AcrA and AcrB, respectively [91].

**2.2.6 CusCFBA**

CusCFBA system is the only Hevay Metal Efflux (HME) RND pump identified in *E. coli* till date [91]. This *cus* gene encoded protein mediates resistance to copper ($Cu^{+}$) and silver ($Ag^{+}$) by

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cation efflux [105]. However, this pump system has been reported to exhibit resistance against fosfomycin [91, 94], dinitrobenzene, dinitrophenol, and ethionamide [106].

### 2.2.7 EmrE and SugE

EmrE and SugE efflux pump are included in small multidrug resistance family (SMR). They consist of 110 amino acids in length which makes them the smallest multidrug resistance transporter proteins [52, 107]. EmerE is known for inducing resistance to lipophilic cations including DNA intercalating dyes (Ethidium Bromide) and quaternary ammonium compounds [52, 107]. Because of its small size, EmrE functions as a homo-oligomer. Studies have shown that over-expression of *sugE* gene confers resistance to toxic quaternary ammonium compounds [108].

### 2.2.8 MdfA

MdfA multidrug Efflux pump, also known as CmlA or Cmr, is a chromosomally encoded transporter protein which belongs to Major Facilitator Superfamily (MFS) [109]. *mdfA* codes for the putative membrane protein MdfA, which contains 410amino acid residues [110]. This pump is known to confer resistance against fluoroquinolone [111] and chloramphenicol.

### 2.2.9 MacAB-TolC

In mammals, ABC transporters are considered to be leading drug efflux systems responsible for multi-drug resistance in cancer cells [52, 112]. MacA and MacB belong to ABC-type (ATP-binding cassette) efflux pump family and mediate antibiotic resistance by ATP hydrolysis mechanism [52, 112]. Both MacA and MacB genes are located in a single operon which is suppressed by PhoPQ system and activated by heat shock sigma factor $\sigma^{32}$ [113-115]. MacAB-TolC transorter system have been found to be potent transporter of macrolide antibiotics [115].

## 3. Plants as a potential source of inhibitors of bacterial efflux pump

The importance of the medicinal plants is determined by the demand of three fourth of the world's population that depend only on plants as the source of medicine. Use of herbal medicines has increased in recent years due to the fact that they are cheap, readily available and effective, as well as the high cost of industrialized medicines, lack of public access to medical and pharmaceutical care, and the side effects caused by synthetic medicines [116]. Plants produce various antibacterial metabolites which constitute their chemical defense system to protect themselves from microbes present in the environment [117]. Various plant extracts or

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phytochemicals have been identified to naturally act as a potential source of bacterial Efflux pump inhibitors (EPIs). Efflux pump inhibitors employ a strategy to block the route of Efflux pumps thereby increase the concentrations of intracellular antibiotics which can then easily reach their target sites to inhibit the efflux pump activity [118]. The plant-derived Efflux pump inhibitors have been reported to help in regaining the activities of existing antibiotics [119]. A combination of Efflux pump inhibitors and antibiotics may increase the intracellular concentration of antibiotics by decreasing the efficiency of bacterial Efflux pumps and thereby reducing the frequency of development of resistant mutant strains [120]. Natural plant extracts have been found to be an important source of secondary metabolites such as terpenoids, tannins, alkaloids and flavonoids that possess antimicrobial properties [121]. Some plant extracts or compounds have also been classified as antibiotic modifiers because they can enhance the activity of antibiotic or reverse the antibiotic resistance [121]. Some plant derived Efflux Pump Inhibitors (Figure 1) exhibiting effective inhibition of *Staphylococcus aureus* and in *Escherichia coli* efflux pumps have been presented in Table 3.

### 3.1 *Acer saccharum* Marsh

*Acer saccharum* Marsh belongs to the family *Sapindaceae*. It is known for its phenolic-rich maple syrup extract (PRMSE). The sap was extracted with methanol and reported as efflux pump inhibitor against *Escherichia coli* (ATCC 700928) [122]. The isolated compound 'catechol' was also able to exhibit strong synergy with antibiotics and other phenolic components of PRMSE as well as inhibit EtBr efflux, but to a lesser extent than syrup extracts [122].

### 3.2 *Portulaca oleracea*

*Portulaca oleracea* belongs to the family *Portulacaceae*. It is the most common plant which is used in folk medicine and also used as vegetable in many countries [123]. This plant is a good source of vitamins, β-carotene, omega-3 fatty acids, alkaloids, terpenoids and as well as essential amino acids. Two active compounds linoleic acid and oleic acid are extracted and identified from HSCCC (High speed counter current chromatography) fraction 18 of *Portulaca oleracea* which displayed antibacterial activities in combination with a synergist erythromycin against MRSA RN4220/pUL5054 [123]. Ethidium bromide efflux inhibitory study exposed that linoleic and oleic acids may also hamper the activity of MsrA efflux pump in several methicillin-resistant *Staphlococcus aureus* (MRSA) strains [123].

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### 3.3 *Callistemon citrinus*

*Callistemon citrinus*, an evergreen tree or shrub, is a member of the family *Myrtaceae* and commonly known by the name of 'Crimson Bottle Brush' [3, 124]. However, *Callistemon* spp. is known for harboring insecticidal, antibacterial and antifungal bioactive compounds [125]. Ethanolic leaf extracts of *Callistemon citrinus* exhibits inhibition of bacterial efflux pumps in *Staphylococcus aureus* (ATCC 9144), which may result in the accumulation of Rhodamine 6G within the cell [3, 126, 127].

### 3.4 *Eucalyptus tereticornis* Sm.

*Eucalyptus tereticornis* belongs to the family *Myrtaceae*. Triterpenoid ursolic acid, a precursor for some putative EPIs compound, is isolated from the leaves of *Eucalyptus tereticornis* [126]. 3-O-acetyl-urs-12-en-28-n-butyl ester (UA-5) and 3-O-acetyl-urs-12-en- 28-isopropyl ester (UA-4) are two potential substances produced upon esterification of Triterpenoid ursolic acid which are found to inhibit efflux pumps of *Escherichia coli* (MDREC-KG4) along with tetracycline by binding to different sites such as AcrA, AcrB, MacB, TolC and YojI [126, 128].

### 3.5 *Alkana orientalis*

*Alkana orientalis*, a member of the *Boraginaceae* family is found to inhibit the growth of *Staphylococcus aureus* with its leaf and flower extracts. The flavonoid Sarothrin (5,7,4-trihydroxy-3,6,8-trimethoxyflavone), obtained by Bio-assay guided fractionation of *Alkana orientalis* displayed inhibition of growth of *Mycobacterium smegmatis* (MIC 75 µM) and *Staphylococcus aureus* (MIC > 800 µM) and possessed efflux pump inhibitory activity [129].

### 3.6 *Hypericum olympicum*

*Hypericum olympicum* belongs to the family *Hypericaceae*. The active compound isolated from the aerial part of the plant *Hypericum olympicum* is Olympicin A. This patented acylphloroglucinol has been found to be active against NorA Efflux pump of *Staphylococcus aureus* 1199B strain resulting in a healthier accumulation of 14C-enoxacin [130].

### 3.7 *Ipomoea muricata* (L.) Jacq.

*Ipomoea muricata* belongs to *Convolvulaceae* family. Lysergol, a clavine alkaloid, isolated from Ipomoea muricata (L.) Jacq. whose derivatives were tested against the sensitive (CA8000) and resistant (MTCC1652 and KG4) *Escherichia coli* strains to determine their potential inhibitory

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activities of the efflux pump. Lysergol and its derivatives have been reported to inhibit the ABC pump YojI of *Escherichia coli* [131].

### 3.8 *Capsicum annuum*

*Capsicum annuum* is a member of *Solanaceae* family. Capsaicin is a novel P-glycoprotein inhibitor of NorA Efflux pump in *Staphylococcus aureus*. It has been found to effectively reduce the invasiveness of *S. aureus* by inhibiting NorA Efflux pump activity when tested on NorA overexpressing strain of *Staphylococcus aureus* 1199B [132].

### 3.9 *Persea lingue*

*Persea lingue* belongs to *Lauraceae* family. Kaempferol-3-O-a-L (2, 4-bis-E-p-coumaroy) rhamnoside is produced upon bioassay-guided fractionation of the ethanolic extract of *Persea lingue* [133]. This active compound has been found to effectively inhibit NorA efflux pump-mediated efflux of Ethidium bromide (EtBr) in *S. aureus* and potentiate ciprofloxacin activity [133].

### 3.10 *Artemisia absinthium*

*Artemisia absinthium* belongs to the family *Asteraceae*. It is reported that chloroform extracts of *Artemisia absinthium* leaves inhibit the growth of *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus* in presence of Berberine, but ineffective alone [134]. An active component 4',5'-O-dicaffeoylquinic acid (4',5'-ODCQA) from *Artemisia absinthium* is reported to act as an efflux pump inhibitor and docking studies have revealed that CQAs preferentially bind to Major Facilitator Superfamily efflux systems [134].

### 3.11 *Scutellaria baicalensis*

*Scutellaria baicalensis* is a member of *Lamiaceae* family. The active compound Baicalein, derived from *Scutellaria baicalensis*, has been reported to significantly restore the activity of β-lactam antibiotics and tetracycline against Methicillin-Resistant *Staphylococcus aureus* (MRSA). It is observed that Baicalein can effectively inhibit MRSA pyruvate kinase and reverse the ciprofloxacin resistance of MRSA, possibly by inhibiting the activity of NorA efflux pump *in vitro* [135].

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### 3.12 *Wrightia tinctoria*

*Wrightia tinctoria* belongs to *Apocynaceae* family. It is a rich source of alkaloids, flavonoids, saponins, tryptophan, indoxyl yielding O-glycoside(s), phenolics, isatin tryptanthrin, anthranilate, β-isatin, rutin, indigotin, wrightial, indirubin and sterols [136]. The active component indirubin isolated from the chloroform extract of *Wrightia tinctoria* R. Br. leaves has been found to be effective against clinically important Gram-positive and Gram-negative bacteria [137]. Indirubin exhibits significant antibacterial activity against both the type strain and drug-resistant *S. aureus* [137]. It potentiates the action of ciprofloxacin synergistically by probably inhibiting the activity of NorA efflux pump [137].

### 3.13 *Alpinia hainanensis* K. Schum (Seeds)

*Alpinia hainanensis* K. Schum plant belongs to family *Zingiberacea*. The monoterpene (-)-a-pinene of the essential oil derived from the seeds of *Alpinia hainanensis* is the first potent EPI reported to date against *C. jejuni* [138]. Promising results were obtained when the *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) strains were treated with the essential oil obtained by the steam distillation of the ethanolic extract of the plant [139].

### 3.14 *Baccharoides adoensis* (Sch.Bip. ex Walp.) H.Rob (Leaves)

*Baccharoides adoensis* (Sch.Bip. ex Walp.) H.Rob is a member of *Compositae/Asteraceae* family. An ethanolic extract of the leaves of this plant has been reported to exhibit a strong EPI action against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 9144 [3].

### 3.15 *Digitalis lanata* Ehrh. (Leaves)

*Digitalis lanata* Ehrh is a member of the family *Plantaginaceae*. The leaves of *Digitalis lanata* Ehrh contain a cardiac glycoside Lanatoside C [126]. This compound has been found as potential EPI in sensitive *E. coli* and *P. aeruginosa* harboring the efflux pumps AcrAB-TolC and MexAB-OprM, respectively [126]. Cardiac glycosides are known for inhibiting $Na^+$-$K^+$-ATPase and increasing intracellular $Ca^{2+}$ concentration [140, 141] so a probable active interaction between ATP-dependent efflux pump and ATPase can be predicted [18].

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### 3.16 *Glycine max* (L.) Merr. (Fruit)

*Glycine max* (L.) Merr. belongs to family *Leguminosae*. It is the source of isoflavone Daidzein which has been evaluated as a potential EPI against *E. coli* (AcrAB-TolC) and *P. aeruginosa* (MexAB-OprM) [18, 126]. It is reported to suppress P- glycoprotein expression and thereby increasing the drug sensitivity of human cervical carcinoma KB-V1 cells [18, 126, 142].

### 3.17 *Zanthoxylum capense* (Thunb.) Harv. (Root)

*Zanthoxylum capense* (Thunb.) Harv. is included in the family *Rutaceae*. Some compounds such as Oxychelerythrine and Ailanthoidiol diacetate, derived from the methanol extract of the root of *Zanthoxylum capense* (Thunb.) Harv., have been reported to increase the bacterial susceptibility [143].

### 3.18 *Momordica balsamina* L. (aerial parts)

*Momordica balsamina* L. is a member of *Cucurbitaceae* family. Six cucurbitane-type triterpenes derived from the methanol extract of *Momordica balsamina* L. have been evaluated for their EPI activity against *Enterococcus faecalis* ATCC 29212 and MRSA $COL_{OXA}$ cells [126, 144] by assessing EtBr accumulation inside cells. Some of them have been also tested against *E. coli* AG100 wild-type strain and AcrAB-TolCE overexpressing *E. coli* AG100TET8 [144].

### 3.19 *Rosmarinus officinalis* L. and *Salvia officinalis* L. (leaves)

*Rosmarinus officinalis* L. and *Salvia officinalis* L. both are included in the family *Lamiaceae*. Carnosic acid, a natural diterpene, present in these two plants have been assessed for EPI activity against the MDR strain *S. aureus* 1199B [126, 145, 146]. It has been presumed that Carnosic acid can act as an Efflux pump modulator by dissipation of the membrane potential [146].

## 4.0 Discussion

Mechanism of active efflux pumps is the most significant one among all the antibiotic resistance development mechanisms in different bacteria. Particularly six most important groups of nosocomial and antibiotic-resistant bacteria are in the focus of study at present namely *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter sp*. (ESKAPE) [147]. The continuous evolution of the harmful and deadly microorganisms makes them resistant to the already available first line of drugs which finally generates the trait of antibiotic resistance. Therefore,

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there is an urgent need for the search of an alternative treatment against multi-drug resistant strains [7].

The medicinal properties of different plants are defined by its content of active phytochemicals. Phytochemicals are chemical compounds that are found naturally in plants. They can be divided into two main categories: (a) Primary constituents that include common sugars, amino acids, proteins and chlorophyll etc. and (b) Secondary constituents consisting of alkaloids, flavonoids, terpenoids, tannins, saponins, various phenolic compounds, essential oils etc. [148, 149]. Phenols and flavonoids contribute towards antioxidant properties of the plant [150]. Most of the phytochemicals are known for their action as insecticidal, antibacterial, antifungal and anticonstipative activities etc.

Some efflux pump inhibitors with plant source have been patented. A tetrandrine based efflux pump inhibitors regain the activities of fluoroquinolones in *Escherichia coli* [126]. Liquiritin has been reported as an efflux pump inhibitor against fluoroquinolone resistance in *Escherichia coli* [126], phenylpropanoids as efflux pump inhibitor against *Mycobacteria* [126] and geraniol served as an efflux pump inhibitor against *Enterobacter aerogenes* [151]. As per the reports of Si, *et al*., 2008, it has been revealed that the administration of fluoroquinolones is able to be more active against broad-spectrum beta-lactamase producing *Escherichia coli* when is a combination with oregano essential oil [152]. In a similar way, terpenoids derived from plants also show similar synergistic effect with antibiotics [153, 154]. Flavonoids have also shown to be effective against *Klebsiella pneumonia* (multi-drug resistant) while it is given with antibiotics [155]. Cefotaxime is another drug which was also demonstrated to have enhanced activity when it combines with aqueous extract and gallotanin extract of *Terminalia chebula* and it was shown to have potent inhibitory activity against efflux pumps of multi-drug resistant *Escherichia coli* [156, 157]. Phenylalanine-arginine b-naphthylamide (PAbN) was the first reported efflux pump inhibitor (EPI) which showed potential efflux inhibitory effect against AcrAB-TolC in *Escherichia coli* [158]. Its mechanism for inhibiting these pumps was postulated as being a Resistance-Nodulation-Division (RND) substrate and therefore competes with the antibiotic for the pocket site of efflux pump [23]. It is also reported that efflux pumps are involved in the down-regulation of quorum sensing signals with bacteria which can adapt very quickly to environmental changes [23]. The advantage of administration of an efflux pump inhibitor is not

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only the diminished drug efflux but also the reduction of inherent resistance and selected resistant mutants. However, it is not essential for efflux pump inhibitor to be an antimicrobial compound [7].

## 5.0 Conclusion

Antibiotic resistance property of bacteria has been a major threat to human health since long time and bacterial efflux pumps are responsible for imparting resistance to many antibiotics. Therefore, in depth study and research on Efflux Pump Inhibitors (EPI) from plant resources urges immediate attention. Even though many efflux pump inhibitors have been identified, it needs to overcome their toxicity and bioavailability property to further introduce it for clinical studies. Besides newly efflux pump inhibitors have to be evaluated very consciously in order to prevent many side effects and toxicities in the human body. Recent studies on efflux pump inhibitors have also examined that Gram-negative bacteria are being given more priority than Gram-positive bacteria. This is because Gram-negative bacteria have evolved extra effective barriers outside the peptidoglycan layer to prevent the entry of most amphipathic compounds like cationic and anionic whereas Gram-positive bacteria have only a single membrane. Therefore, there is a need to explore new plant sources for efflux pump inhibitors against Gram-negative bacteria. The wide distribution of efflux genes in *Staphylococcus aureus* makes efflux pump inhibitors promising therapeutic agents against bacterial infection. Efflux pump plays a significant role in development of antibiotic or drug resistant microbial strains. Consequently, it prevents proper medication for several chronic nosocomial diseases. Therefore, understanding the underlying mechanisms of different efflux pump and development of effective inhibitors is the goal of near future. Phytochemicals have shown promising results as efflux pump inhibitors. Therefore, further studies are required to understand various mode of action of phytochemicals as efflux pump inhibitors.

**Acknowledgement**

The authors are acknowledged to their respective departmental colleagues for all support and help.

**Conflict of Interest:**

Authors have no conflict of interests.

**Funding:**

The following study includes no funding from any organization.

**Ethical Approval:**

Not Required.

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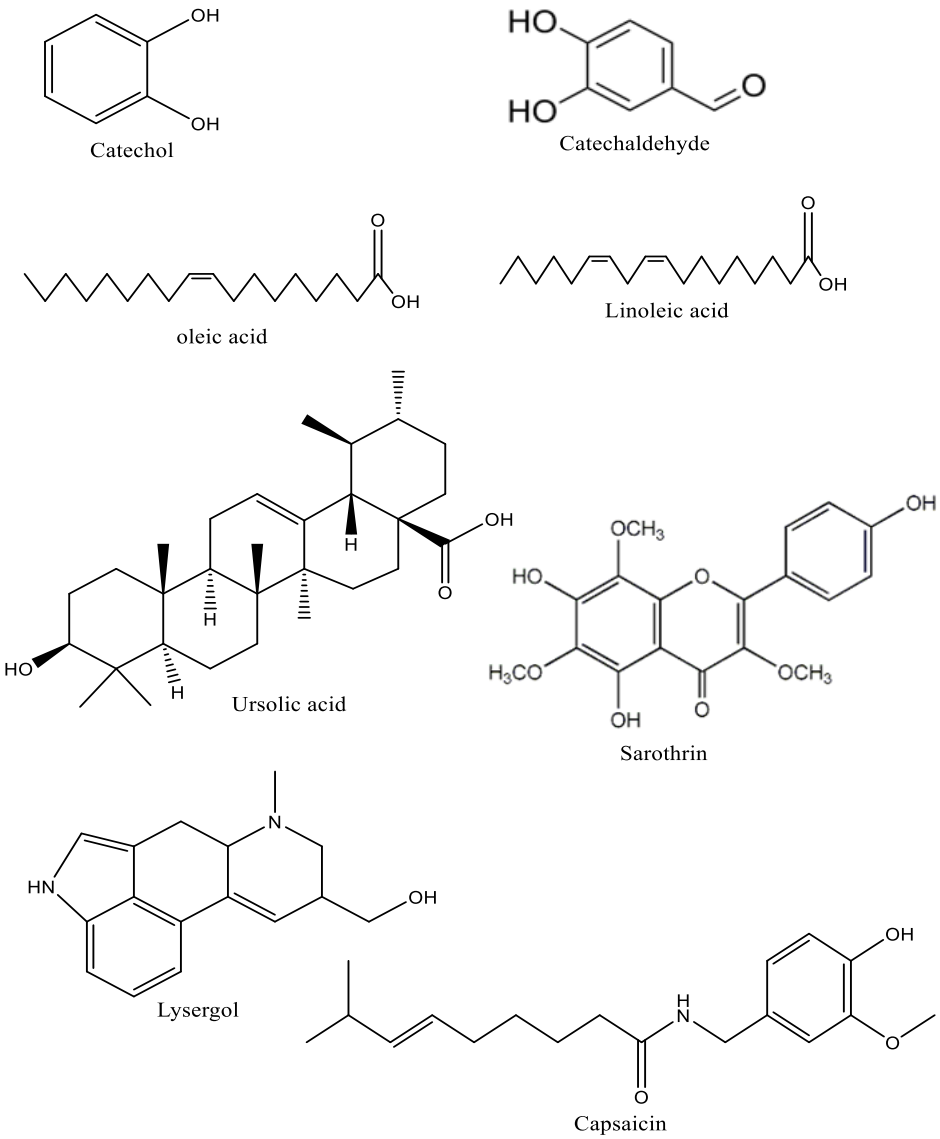

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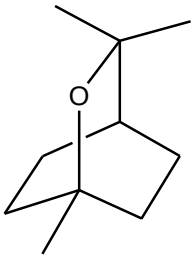


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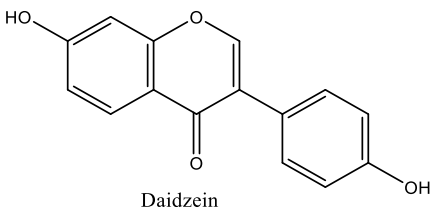


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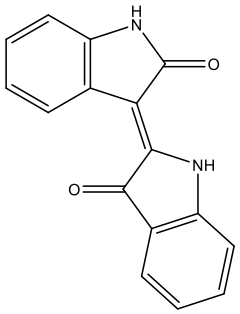


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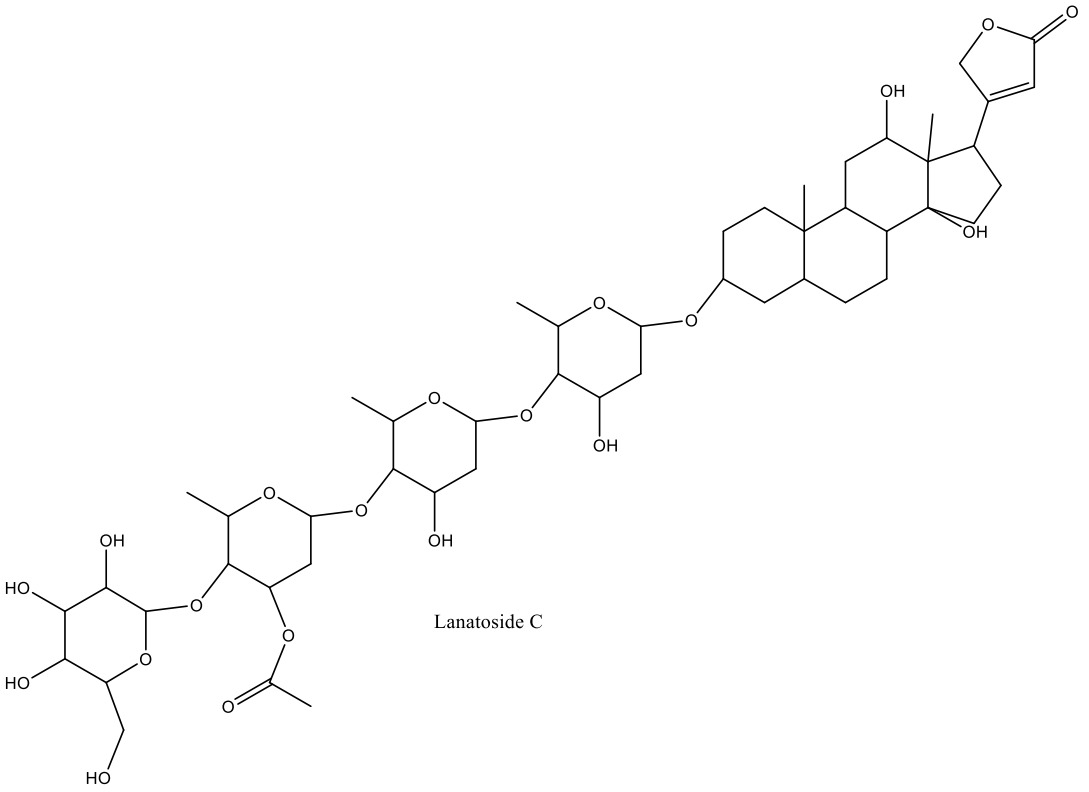


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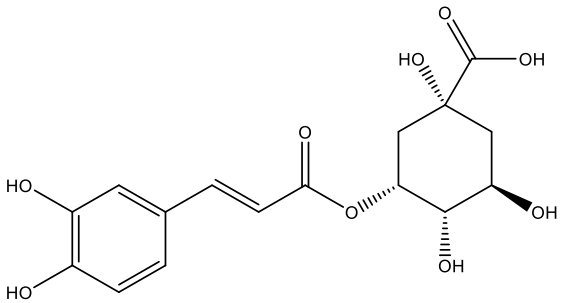


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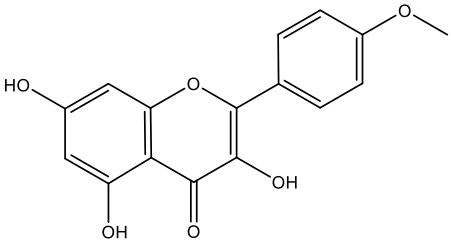


Kaempferol

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**Figure 1.** Chemical structures of different plant-derived compounds identified as potent bacterial efflux pump inhibitors.

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**Table 1.** List of different bacterial species harbouring various families of efflux pumps along with tested antibiotics.

| Family Of Efflux Pumps | Substrate Nature | Used Antibiotics | Efflux-Pump Containing Bacteria | Reference |
|---|---|---|---|---|
| **SMR** | Lipophilic, Multicationic | Erythromycin, Tetracycline, Sulfadiazine | *Staphylococcus aureus* and *Acinetobacter baumannii* | [159] |
| **RND** | Charged, Aphiphilic | Fluoroquinolone, Tetracycline, Erythromycin, Rifampicin, β- lactam, Chloramphenicol, Fusidic acid, Aminoglycosides | *Escherichia coli* and *Pseudomonas aeruginosa* | [160], [161], [21] |
| **MFS** | Mono or Dicationic, Amphiphilic | Fluoroquinolone, Tetracycline, Erythromycin, Lincosamides, Pristinamycin, Rifampicin, Chloramphenicol, Aminoglycosides | *Staphylococcus aureus* and *Escherichia coli* | [21] |
| **ABC** | Cationic, Amphiphilic neutral | Teracycline, Fluoroquinolone, Macrolids, Rifanpicin, Lincosamides,Chloramphenicol, Aminoglycosides | *Staphylococcus aureus* and *Lactococcus lactis* | [162] |
| **MATE** | Cationic low molecular weight | Norfloxacin, Amioglycosides, Fluoroquinolone | *Staphylococcus aureus*, *Escherichia coli* and *Vibrio* | [163] |

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| | | | | |
|---|---|---|---|---|
| | | | *parahaemolyticus* | |

**Table 2.** List of major efflux pumps reported to be present in *Staphylococcus aureus* & *Escherichia coli*

| ***Microorganism*** | ***Major Efflux Pumps*** | ***Reference*** |
|---|---|---|
| *Staphylococcus aureus* | QacA, QacB, LmrS, MdeA, QacG, QacH, QacJ, NorA, NorB, NorC, NorD, TetA(K), Tet38, SdrM and Mef(A). | [52] |
| *Escherichia coli* | AcrA-TolC, AcrB-TolC, EmrE, SugE, MdfA, MacA and MacB | |

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**Table 3.** Some plant derived Efflux Pump Inhibitors (EPIs) exhibiting effective inhibition of *Staphylococcus aureus* and in *Escherichia coli* efflux pumps.

| Name of plants and parts used | Active against | Name of phytochemicals as efflux pump inhibitor | Synergist | Efflux pump inhibited | References |
|---|---|---|---|---|---|
| *Acer saccharum Marsh* (Sap) | *E. coli* | Phenolic-Rich-Maple-Syrup Extract (PRMSE): Catechol and Catechaldehyde | Ciprofloxacin | - | [122], [126] |
| *Portulaca Oleracea* L. | *S. aureus* | Linoleic and Oleic acid | Erythromycin | MsrA | [123] |
| *Digitalis lanata*Ehrh. (leaves) | *E. coli* | Lanatoside C | Carbenicillin | AcrAB-Tol C | [18], [126] |
| *Callistemon citrinus and Vernonia adoensis* (leaves) | *S. aureus* | Eucalyptol (1,8- cineole) | Rhodamine 6G | - | [3], [126] |
| *Eucalyptus tereticornis*Sm. (leaves) | *E. coli* | Ursolic acid derivatives | Tetracycline | AcrA | [128] |
| *Glycine max* (L.) Merr. (fruit) | *E. coli* | Daidzein | Carbenicillin | Mex-AB, Opr_M, AcrAB- TolC | [18] |
| *Alkanaorientalis* (Leaves and flowers) | *S. aureus* | Sarothrin | - | NorA | [129] |
| *Hypericum olympicum*(aerial parts ) | *S. aureus* | Olympicin A | - | NorA | [130] |

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| *Ipomoea muricata* (L.) Jacq. (seeds) | *E. coli* | Lysergol and its derivatives | Tetracycline | YojI | [131] |
| *Capsicum annuum L.* (fruit) | *S. aureus* | Capsaicin | Ciprofloxacin | NorA | [132] |
| *Persealingue* (Leaves) | *S. aureus* | Kaempferol | - | NorA | [133] |
| *Artemisia absinthium* | *S. aureus* | Caffeoylquinic acids | Berberine | NorA | [134] |
| *Scutellariabaicalensis* | *S. aureus* | Baicalein | Ciprofloxacin | NorA | [135] |
| *Wrightiatinctoria* (Leaves) | *S. aureus* | Indirubin | Ciprofloxacin | NorA | [137] |